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Rescue of Recombinant Marburg Virus from cDNA Is Dependent on Nucleocapsid Protein VP30

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Here we report recovery of infectious Marburg virus (MARV) from a full-length cDNA clone. Compared to the wild-type virus, recombinant MARV showed no difference in terms of morphology of virus particles, intracellular distribution in infected cells, and growth kinetics. The nucleocapsid protein VP30 of MARV and Ebola virus (EBOV) contains a Zn-binding motif which is important for the function of VP30 as a transcriptional activator in EBOV, whereas its role for MARV is unclear. It has been reported previously that MARV VP30 is able to support transcription in an EBOV-specific minigenome system. When the Zn-binding motif was destroyed, MARV VP30 was shown to be inactive in the EBOV system. While it was not possible to rescue recombinant MARV when the VP30 plasmid was omitted from transfection, MARV VP30 with a destroyed Zn-binding motif and EBOV VP30 were able to mediate virus recovery. In contrast, rescue of recombinant EBOV was not supported by EBOV VP30 containing a mutated Zn-binding domain.

The filoviruses Marburg virus (MARV) and Ebola virus (EBOV) cause a severe hemorrhagic fever in humans and nonhuman primates with extraordinarily high fatality rates. MARV was first isolated in 1967, when 31 laboratory workers in Germany and Yugoslavia handling MARV-infected African green monkeys imported from Uganda became ill. Despite aggressive supportive treatment, seven of the patients died (14, 23). The largest MARV outbreak to date took place from 2004 to 2005 in Angola, when 252 people became infected. The case fatality rate of this outbreak was 91%.

The nonsegmented negative-sense RNA genome of MARV is 19,111 bases in length and encodes seven proteins (9). Four of these proteins (NP, VP35, L, and VP30) constitute the nucleocapsid complex (1). NP, VP35, and L are sufficient to mediate viral transcription and replication in a MARV-specific minigenome system, while the fourth component of the nucleocapsid complex, VP30, acts as a transcription activator for EBOV (17, 18, 28). Hence, the role of VP30 in the life cycle of MARV VP30 has not yet been determined. It has been reported that MARV VP30 interacts with NP-derived inclusions, indicating that VP30 might be involved in nucleocapsid maturation (16). RNA interference-based down-regulation of VP30 in MARV-infected cells resulted in significant reduction of all viral proteins, suggesting an important role for VP30 in viral replication and/or transcription (10). EBOV VP30 contains a Cys_3-His motif comprising amino acids 68 to 95 which was shown to bind zinc ions. The integrity of the Zn-binding motif was crucial for the function as a transcriptional activator but not for the interaction with NP-derived inclusion bodies. Sequence comparison revealed that this motif is also present in MARV VP30 (amino acids 74 to 99) (15). The only other nonsegmented negative-strand RNA viruses possessing a fourth nucleocapsid protein are the pneumoviruses. For human respiratory syncytial virus, it was shown that the M2-1 protein serves as an elongation and antitermination factor during transcription (6, 8, 13). Interestingly, M2-1 contains a Zn finger motif similar to the motif found in VP30 which was shown to be essential for the function of the protein (12).

To study aspects of filovirus replication and transcription without biosafety level 4 containment, minigenome systems were established for MARV and EBOV (2, 11, 17, 18). However, a full-length rescue system is desirable to investigate all aspects of the viral life cycle in an authentic context. Rescue of negative-strand RNA viruses from cDNA was facilitated by using the antigenomic instead of the genomic sequence (22). Since then, full-length rescue systems have been established for several *Mononegavirales* (for reviews, see references 7 and 20), including EBOV (19, 26). These systems allow the specific mutation of proteins of interest (19, 26) or introduction of foreign reporter genes like enhanced green fluorescent protein (25).

In this study, we present a system which allows the recovery of infectious MARV entirely from cDNA. Using this system, the role of VP30 for the rescue of recombinant MARV was investigated.

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Cloning of the full-length MARV clone. The complete genomic sequence of MARV strain Musoke was determined and submitted as a reference sequence to GenBank (accession number DQ217792). A set of five cassettes using a pBlueScript II KS(+) backbone (Stratagene) was designed which could be combined to generate a full-length cDNA of the complete MARV antigenome termed pMARV(+). Reverse transcrip-

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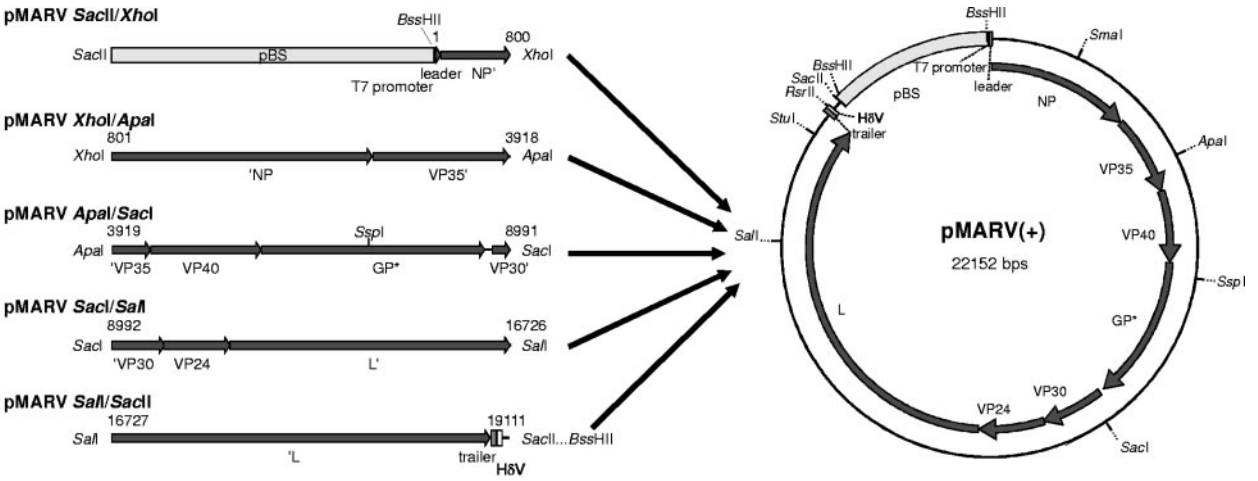

FIG. 1. Cloning strategy for the full-length clone. (Left) Schematic drawing of the cassettes used for cloning. The numbers indicate nucleotide positions in the MARV genome (strain Musoke; GenBank accession number DQ217792). The MARV-specific sequence was obtained either by RT-PCR with viral RNA as the template or PCR assay of already existing plasmids derived from viral RNA. The SspI site shown in pMARV ApaI/SacI serves as a genetic marker. Restriction sites flanking the cassettes were used for construction of full-length pMARV(+) (right).

tion (RT)-PCR with viral RNA as the template and PCR using already existing plasmids containing MARV-specific sequences were used to generate 2.1- to 7.8-kb fragments flanked by unique restriction sites (Fig. 1). All five MARV-specific plasmids were digested with the respective enzymes shown in Fig. 1 and ligated to yield pMARV(+). The correct sequence was verified by automated sequencing on a MegaBACE sequencer (Amersham). To discriminate recombinant from wild-type virus, a silent mutation (viral RNA: $A_{6225\rightarrow}U$) was introduced into the GP gene by QuikChange mutagenesis (Stratagene), generating an additional SspI restriction site at nucleotide (nt) 6220. Furthermore, the first nucleotide of the MARV-specific sequence was mutated (A→G) to enhance the T7 RNA polymerase promoter activity (21). Prior to cloning of the full-length clone, the effect of the A-to-G substitution on replication and transcription activity was tested by using positive-sense minigenomes containing the mutation. It was observed that this mutation did not affect replication and transcription efficiency (data not shown). The full-length clone pMARV(+) is flanked by a T7 RNA polymerase promoter upstream of the leader and a hepatitis delta virus ribozyme downstream of the trailer (29). Transcription of the plasmid by the T7 RNA polymerase resulted in a full-length antigenomic RNA.

Rescue of recombinant MARV. To generate recombinant MARV (recMARV), BSR T7/5 cells (4) were grown in 25-cm^2 flasks to 60 to 80% confluence and transfected with plasmids coding for the MARV nucleocapsid proteins NP, VP35, L, and VP30 (17) and the full-length construct pMARV(+) as described in reference 27. Titration experiments performed with the full-length clone as the template showed that the optimal amounts of the plasmids were 1.0 μg of pT/L_M, 0.5 μg of $pT/VP35_M$, 0.5 μg of pT/NP_M, 0.1 μg of $pT/VP30_M$, and 4.0 μg of pMARV(+). Additionally, 1.0 μg of pC-T7Pol, a plasmid carrying the T7 RNA polymerase, was transfected (19). In contrast to the MARV-specific minigenome system, in which 0.1 μg of pT/NP_M input DNA was found to be optimal, the amount of NP-specific DNA had to be increased to 0.5 μg to generate recombinant virus (17). Five to six days later, transfected BSR T7/5 cells were scraped off in the medium and mixed with Vero cells. Omitting this step did not result in reproducible rescue of recMARV, most likely because the transfected cells died before recombinant virus was produced. After 6 to 8 days, cells were lysed, the lysates were cleared of debris by a short centrifugation step, and the supernatants were used to infect fresh Vero cells. Cells infected with wild-type (wt) MARV or recMARV showed a prominent and similarly pronounced cytopathic effect (CPE) on day 6 postinfection (p.i.) (Fig. 2A). To further characterize the recombinant virus, 50% tissue culture infectious dose ($TCID_{50}$) assays, RT-PCR, immunofluorescence analysis of infected cells, and electron microscopy studies with purified virus were performed and compared with wt MARV. Viral titers of supernatants from infected cells, which had shown comparable CPEs, were virtually identical as determined by $TCID_{50}$ analysis (Fig. 2B). Briefly, Vero cells in 96-well plates were infected with serial dilutions of the virus in quadruplicate and incubated until the CPE remained constant. At 12 days p.i., the CPE was evaluated and the $TCID_{50}$ titer calculated using the Spearman and Karber method. The genetic tag was verified by RT-PCR on RNA obtained from sucrose cushion-purified virions. The supernatant of infected Vero cells was pelleted through a 20% sucrose cushion (wt/vol) in TNE (10 mM Tris-HCl, pH 7.4; 0.15 M NaCl; 2 mM EDTA), and the pellet was resuspended in 600 μl of RLT buffer (RNeasy kit; QIAGEN). Viral RNA was purified following the manufacturer's instructions and first reverse transcribed (Omniscript; QIAGEN) using a primer targeting nt 5890 to 5910 (GP gene) of negative-strand MARV RNA. The first-strand cDNA was subjected to PCR to amplify nt 5890 to 6521, which contained the additional SspI restriction site. After purification and digestion with SspI, two fragments (331 and 301 bp) verified recombinant MARV (Fig. 2C, lane 4), while a single band at 632 bp indicated wt MARV (Fig. 2C, lane 2). No plasmid DNA was present, since omitting the reverse transcriptase from the reaction did not produce any signal (Fig. 2C, lane 5). The presence of infectious virus was

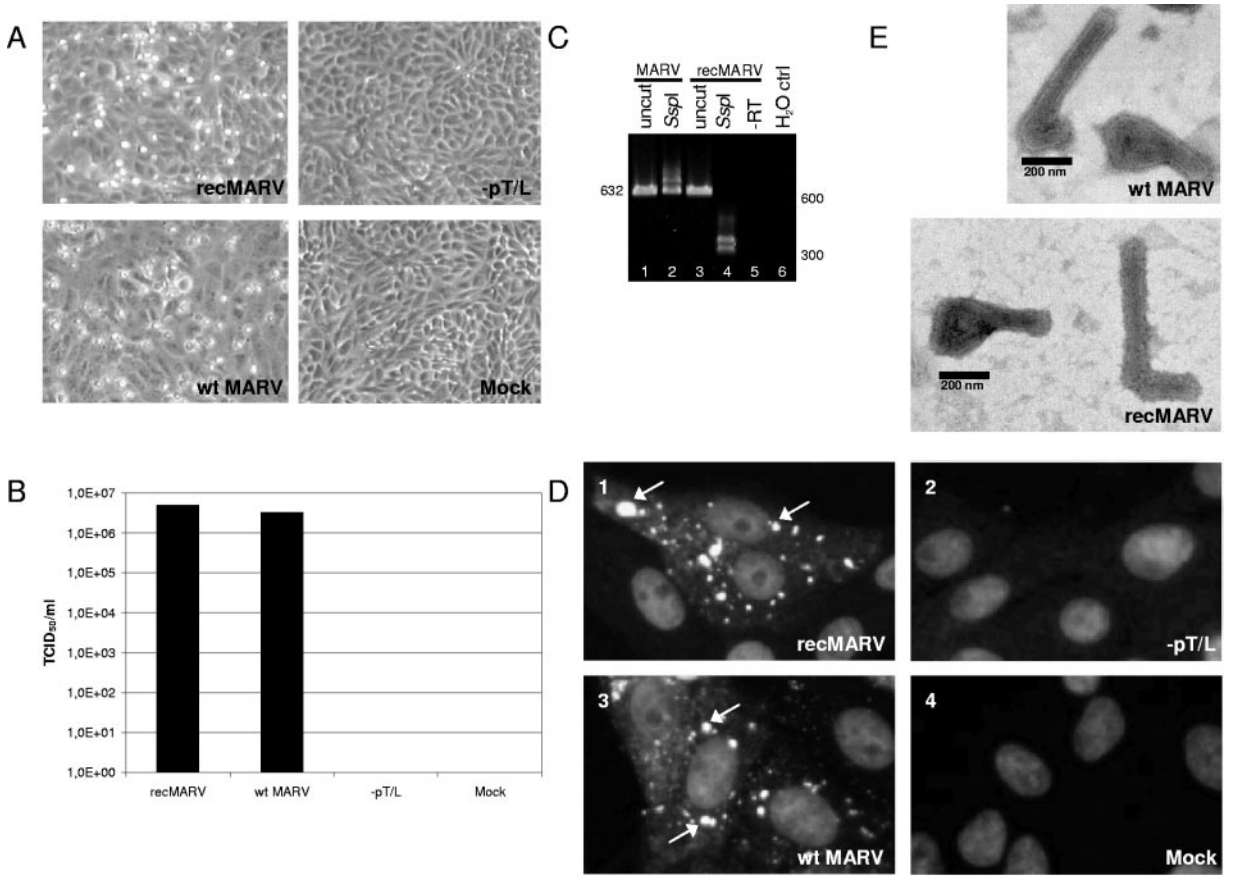

FIG. 2. Characterization of recMARV. Virus was recovered from transfected BSR T7/5 cells mixed with Vero cells as described in the text. Vero cells in 25-cm^2 flasks were inoculated with the lysate of the mixed culture and checked for CPE daily. As a control, the plasmid encoding the L gene was omitted from the tranfection (−pT/L). (A) Light microscopic picture of CPE caused by wt MARV and recMARV on day 6 p.i. (B) Determination of viral titers. Vero cells in 96-well plates were infected with serial dilutions of the virus to determine the viral titer. After 12 days, the CPE was evaluated and the $TCID_{50}$ calculated. (C) Confirmation of the genetic marker. Virus from the supernatant of infected Vero cells was purified through a sucrose cushion at day 6 p.i. Viral RNA was isolated, reverse transcription was performed (except −RT, lane 5), and first-strand cDNA was subjected to PCR. The 632-bp product (lanes 1 and 3) was purified and digested with SspI where stated. Two bands at 301 and 331 bp, respectively, indicated the presence of the genetic tag in recMARV GP gene (lane 4). ctrl, control. (D) Immunofluorescence analysis of Vero cells infected with recMARV or wt MARV. Vero cells were grown on glass coverslips and infected with wt MARV or recMARV at a multiplicity of infection of 1. At 48 h p.i., virus was inactivated in 4% paraformaldehyde overnight. Immunofluorescence analysis was performed using a monoclonal mouse anti-MARV NP antibody and rhodamine-conjugated goat anti-mouse immunoglobulin G; nuclei were stained with DAPI. Arrows indicate viral inclusion bodies. (E) Electron microscopic pictures of purified virions. Virus was collected from the supernatant of infected Vero cells at 6 days p.i. and purified through a sucrose cushion. Virus was inactivated overnight in 4% paraformaldehyde and prepared for electron microscopy as previously described (26). Pictures were taken on a Zeiss 109 electron microscope at a magnification of ×50,000.

further characterized by immunofluorescence analysis. Vero cells were grown on glass coverslips and infected with recMARV or wt MARV or incubated with medium (mock) or the sample in which the L plasmid was omitted (−pT/L). At 48 h p.i., the samples were inactivated in 4% paraformaldehyde overnight. Cells were incubated with a monoclonal antibody raised against MARV NP (1:100 dilution). As a secondary antibody, a rhodamine-conjugated goat anti-mouse antibody (1:200 dilution; Dianova) was used. Additionally, nuclei were stained with 0.1 μg/ml 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI). As shown in Fig. 2D, the typical inclusion bodies formed by the nucleocapsid proteins were observed in cells infected with either recMARV or wt MARV. No specific staining was observed in mock-infected cells or the sample transfected without the L gene. Lastly, virions were purified through a sucrose cushion as described above, inactivated in 4% paraformaldehyde overnight, and analyzed by electron microscopy as described by Volchkov et al. (26). Recombinant MARV showed no morphological differences compared to wt MARV (Fig. 2E). Taken together, recMARV could be rescued with plasmids encoding the nucleocapsid proteins VP30, VP35, L, and NP and was indistinguishable from wt MARV in terms of morphology, infectivity, viral titer, and immunofluorescence staining.

Integrity of the zinc-binding motif Cys_3-His within VP30 is not needed for interaction with NP but for transcriptional activity in an EBOV-specific minigenome system. Rescue of recMARV was only supported in the presence of all nucleocapsid proteins. When the fourth nucleocapsid protein, VP30, was omitted, it was not possible to generate recombinant MARV (see below and Fig. 4). Similarly, recovery of recombinant EBOV was also dependent on the presence of VP30

(19, 26). Interestingly, the necessity of the fourth nucleocapsid protein M2-1 for virus rescue is different among the pneumoviruses. While human respiratory syncytial virus M2-1 was found to be necessary for production of recombinant virus, recombinant human metapneumovirus lacking the M2-1 open reading frame was successfully recovered in cell culture (3, 5). Concerning MARV, VP30 was not needed for replication and transcription of MARV minigenomes (17). Thus, the question arose of whether VP30 is involved in transcription and/or replication of the recombinant virus or whether it acts as a structural component during nucleocapsid formation.

The Zn-binding motif Cys_3-His of EBOV VP30 was shown to be necessary for transcriptional activity but not for binding to NP (15). First, the importance of the Zn-binding motif of MARV VP30 was examined with regard to binding to NP and transcriptional activity in an EBOV-specific minigenome system (18). The expression plasmid $pT/VP30_{M\ Zn\text{-}finger\ knockout}$ contained two substitutions (genomic positions $G_{9143\rightarrow}C$, $A_{9155\rightarrow}U$, mRNA sense) introduced by QuikChange mutagenesis on $pT/VP30_M$ (17), which resulted in the disruption of the putative Zn-finger motif ($Cys_{92\rightarrow}Ser$, $His_{96\rightarrow}Leu$) (Fig. 3A). It has been shown previously for EBOV VP30 that mutation of the homologous amino acids led to a loss of function as transcription activator, while binding to NP-derived inclusion bodies was not impaired (15). Expression of $VP30_M$ and $VP30_{M\ Zn\text{-}finger\ knockout}$ was verified by Western blot analysis. Therefore, BSR T7/5 cells were transfected with 1.0 and 2.0 μg, respectively, of either $pT/VP30_M$ or $pT/VP30_{M\ Zn\text{-}finger\ knockout}$ and lysed at 2 days posttransfection. $VP30_M$ was detected using a monoclonal mouse anti-$VP30_M$ antibody (1:1,000) and a peroxidase-labeled goat anti-mouse antibody (1:40,000). As shown in Fig. 3C, both constructs were expressed at comparable levels. Immunofluorescence analysis was employed to study the interaction of $VP30_{M\ Zn\text{-}finger\ knockout}$ with NP. BSR T7/5 cells were grown on coverslips and transfected with either 0.5 μg pT/NP_M, 1.5 μg $pT/VP30_M$, 1.5 μg $pT/VP30_{M\ Zn\text{-}finger\ knockout}$, or a combination of NP and VP30 plasmids. After 2 days, cells were fixed and permeabilized. NP was detected as described above, and VP30 was detected using a guinea pig anti-VP30 antibody (1:100) and fluorescein isothiocyanate-conjugated goat anti-guinea pig antibodies (1:200; Dianova). Upon single expression, NP formed inclusion bodies (Fig. 3B, panel 1), whereas $VP30_M$ and $VP30_{M\ Zn\text{-}finger\ knockout}$ were homogenously distributed in the cytoplasm (Fig. 3B, panels 2 and 3). When NP and $VP30_M$ or the mutant $VP30_{M\ Zn\text{-}finger\ knockout}$ were coexpressed, both VP30 variants were redistributed into the NP-derived inclusion bodies (Fig. 3B, panels 4 and 5). These data indicate that an intact Zn-finger motif is not important for binding to NP, as also described for EBOV VP30 (15). The next aim was to determine the functionality of $VP30_{M\ Zn\text{-}finger\ knockout}$ with respect to transcription activation. As mentioned above, MARV VP30 was not required for transcription activation in a MARV-specific minigenome system. However, it has been described previously that MARV VP30 was able to mediate transcription to some extent when used in an EBOV-specific minigenome system. When cells were transfected with plasmids encoding EBOV NP, VP35, L, the EBOV-specific minigenome, and MARV VP30, transcription activation was observed (18). Following the transfection procedure of BSR T7/5 cells described by Weik et al. (27), we replaced $pT/VP30_{EBO}$ with 2.0 μg of either $pT/VP30_M$, pT/

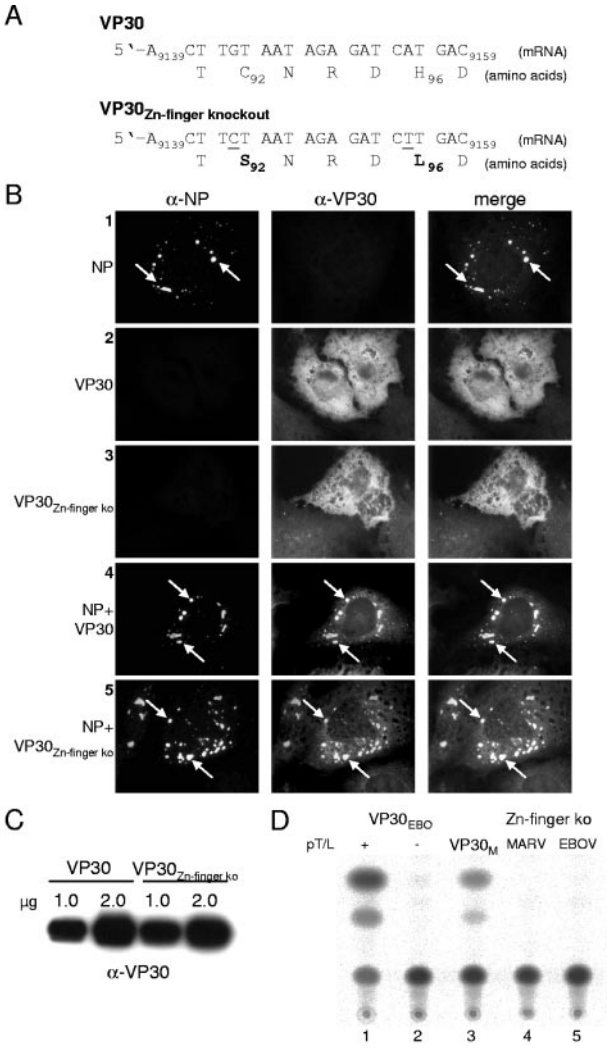

FIG. 3. Localization and functionality of $VP30_{Zn\text{-}finger\ knockout}$. (A) Putative Zn-binding domain of MARV VP30. Two mutations (underlined) were introduced into $pT/VP30_{M\ Zn\text{-}finger\ knockout}$ to disrupt the putative Zn-binding motif. The nucleotide numbers indicate positions in the MARV genome. (B) BSR T7/5 cells were grown on glass coverslips and transfected with pT/NP_M (NP), $pT/VP30_M$ (VP30), and $pT/VP30_{M\ Zn\text{-}finger\ knockout}$ ($VP30_{Zn\text{-}finger\ ko}$) as indicated on the left. At 48 h posttransfection, cells were subjected to immunofluorescence analysis using a monoclonal mouse anti-NP (α-NP) and a guinea pig anti-VP30 (α-VP30) antibody. NP was stained with rhodamine-conjugated goat anti-mouse antibodies and VP30 with fluorescein isothiocyanate-conjugated goat anti-guinea pig antibodies. The arrows indicate typical inclusion bodies formed by NP. (C) Western blot analysis of $VP30_{M\ Zn\text{-}finger\ knockout}$. BSR T7/5 cells were transfected with 1.0 and 2.0 μg, respectively, of either $pT/VP30_M$ or $pT/VP30_{M\ Zn\text{-}finger\ knockout}$ and analyzed by Western blotting at 2 days posttransfection. $VP30_M$ was detected using a monoclonal mouse anti-$VP30_M$ (α-$VP30_M$) antibody and a horseradish peroxidase-labeled goat anti-mouse antibody. (D) Transcriptional activity of $VP30_{M\ Zn\text{-}finger\ knockout}$ using the EBOV-specific minigenome system. BSR T7/5 cells were transfected with plasmids encoding the nucleocapsid proteins of EBOV and the minigenome 3E-5E (27). EBOV VP30 was replaced with 2.0 μg of $VP30_M$ or 2.0 μg of Zn-finger knockout (ko) mutants of the MARV-specific $VP30_M$ ($VP30_{M\ Zn\text{-}finger\ knockout}$) or the EBOV-specific $VP30_{EBO}$ ($VP30_{E\ H90L}$) (15). Cells were lysed after 48 h and subjected to a CAT assay. +, pT/L present; −, pT/L absent.

$VP30_{M\ Zn\text{-}finger\ knockout}$, or $pT/VP30_{E\ H90L}$, an EBOV VP30 mutant with a disrupted Zn-finger motif (15). Transfected cells were lysed on day 2 posttransfection. The used minigenome 3E-5E consists of the 3′ and 5′ ends of the EBOV genome and a chloramphenicol acetyltransferase (CAT) gene as reporter gene (18). Transcription of the minigenome leads to CAT gene expression and was assayed by CAT activity (17). While MARV VP30 exhibited between 10 and 15% activity (Fig. 3D, lane 3), neither of the Zn-finger knockout mutants was able to activate transcription (Fig. 3D, lanes 4 and 5). Hence, the Zn-binding motif of MARV and EBOV VP30 seems to have a similar function, although the details are not yet understood (15).

MARV can be rescued with EBOV VP30 and $VP30_{M\ Zn\text{-}finger\ knockout}$. Next, we examined the role of VP30 in the full-length rescue system. BSR T7/5 cells were transfected in triplicate as described above. Plasmid $pT/VP30_M$ was substituted with either 0.1 μg $pT/VP30_{EBO}$ coding for EBOV VP30 or 0.5 μg $pT/VP30_{M\ Zn\text{-}finger\ knockout}$. Supernatants from mixed BSR T7/5 and Vero cells were used to infect fresh Vero cells, and at 10 days p.i., total cellular RNA was isolated and RT-PCR was performed as described earlier. As mentioned above, no signal was detected when VP30 was omitted from the transfection mixture (Fig. 4A, lanes 2 to 4). These data demonstrate that MARV VP30 is necessary for rescue of full-length virus, although it is not needed for transcription and replication in the minigenome system (17). Strong signals were obtained from all samples transfected with $pT/VP30_{M\ Zn\text{-}finger\ knockout}$ (Fig. 4A, lanes 5 to 7), indicating that the Zn-binding motif was not essential for recovery of recMARV, although it was necessary for transcription activation in the EBOV-specific minigenome system (Fig. 3D). Rescue was also observed in one of three samples when the EBOV VP30 plasmid was transfected (Fig. 4A, lanes 8 to 10). In contrast, it was not possible to generate recombinant EBOV when MARV VP30 was used in an EBOV rescue system (24).

To compare the effect of the VP30 Zn-binding domain on virus recovery between MARV and EBOV, similar experiments were performed by using the EBOV-specific rescue system. BSR T7/5 cells were transfected with plasmids coding for the EBOV nucleocapsid proteins NP, VP35, and L, the EBOV-specific full-length clone pFL-EBOVe+ in positive orientation, EBOV VP30, or the Zn-finger mutant $VP30_{E\ H90L}$ as described earlier (15, 26). Briefly, BSR T7/5 cells were grown overnight in 25-cm^2 flasks to about 60% confluence and transfected with a plasmid mixture containing 4 μg of the full-length plasmid pFL-EBOVe+, 1 μg of $pT/VP35_{EBO}$, 1 μg of pT/NP_{EBO}, 2 μg of pT/L_{EBO}, and 0.2 μg of either $pT/VP30_{EBO}$ or $VP30_{E\ H90L}$ by using Fugene 6 reagent (15, 26). After 6 days, culture medium was collected and used for inoculation of Vero E6 cells. At 6 days p.i., successful rescue was demonstrated by the strong CPE caused by infection with the recombinant EBOV. It is shown in Fig. 4B that virus recovery was only observed in the presence of the nonmutated version of VP30. The mutant $VP30_{E\ H90L}$, however, was not able to support rescue. These data demonstrate that, in contrast to MARV, an intact VP30 Zn-binding domain is essential for rescue of recombinant EBOV, confirming the assumption that VP30 acts as a transcription activator for EBOV but not for MARV. Thus, MARV VP30 might function rather as a structural component that is probably involved in correct formation of the nucleocapsid. However, it has to be noted that even poor replication and transcription of recMARV by support plasmids could lead to generation of infectious virus. Since the recombinant virus contained the nonmutated version of the VP30 gene, it could amplify without restriction, leading to the observed results. Also, it is conceivable that inefficient translation of the antigenome yielded low levels of wild-type VP30. However, this explanation for the observed results is not very likely because if this were the case, rescue should also occur in the absence of VP30. To ultimately clarify the role of the Zn-binding domain for virus rescue, the next step will be to assess the effect of VP30 mutations in the VP30 gene of the full-length genome.

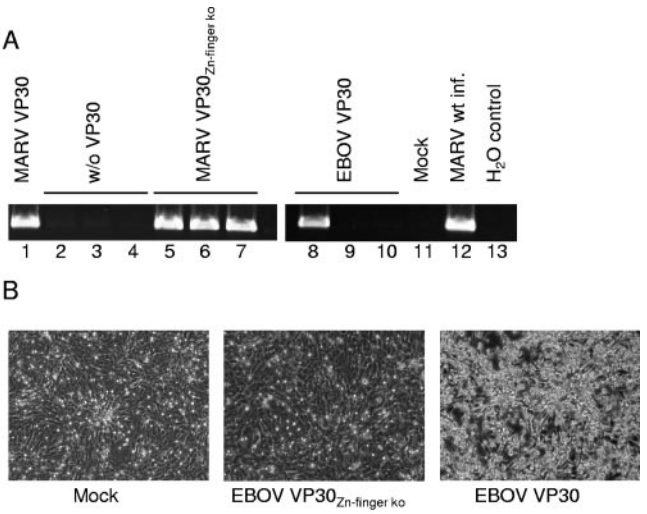

FIG. 4. Influence of VP30 on recovery of recombinant MARV and EBOV. (A) BSR T7/5 cells were transfected with plasmids coding for MARV nucleocapsid proteins NP, L, and VP35, the full-length clone pMARV(+), and pC-T7Pol. A plasmid encoding either MARV VP30 (lane 1), the MARV VP30 Zn-finger mutant ($VP30_{Zn\text{-}finger\ ko}$, lanes 5 to 7), or EBOV VP30 (lanes 8 to 10) was added to the reaction mixture or VP30 was omitted from the transfection (w/o VP30, lanes 2 to 4). Except for the positive control (MARV VP30), transfection was performed in triplicate. At 6 days posttransfection, BSR T7/5 cells were mixed with Vero cells and lysed after an appropriate incubation period. Fresh Vero cells were infected with the supernatants and incubated until day 10 p.i. Cells were lysed, and total RNA was isolated and subjected to a one-step RT-PCR using primers targeting the MARV GP gene (nt 5890 to 6521). Ten percent of the reaction mixture was run on a 2% agarose gel and visualized with ethidium bromide. As controls, cells were infected with MARV (MARV wt inf., lane 12) or not infected (mock, lane 11). (B) BSR T7/5 cells were transfected with plasmids encoding EBOV NP, L, VP35, the full-length clone pFL-EBOVe+, and either $VP30_{EBO}$ or the Zn-finger mutant $VP30_{E\ H90L}$ (EBOV $VP30_{Zn\text{-}finger\ ko}$). At 6 days posttransfection, supernatants were used to infect Vero E6 cells. CPE caused by virus infection was determined after an incubation period of 6 days.

Taken together, we established a T7 RNA polymerase-driven full-length rescue system for the filovirus prototype MARV. This system provides the opportunity to study the role of viral proteins and *cis*-acting elements in replication and transcription of mutant virus and will help us to gain insight in the viral life cycle.

Nucleotide sequence accession number. The complete genomic sequence of MARV strain Musoke was submitted to GenBank under accession number DQ217792.

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REFERENCES

1. **Becker, S., C. Rinne, U. Hofsäss, H.-D. Klenk, and E. Mühlberger.** 1998. Interactions of Marburg virus nucleocapsid proteins. Virology **249:**406–417.
2. **Boehmann, Y., S. Enterlein, A. Randolf, and E. Mühlberger.** 2005. A reconstituted replication and transcription system for Ebola virus Reston and comparison with Ebola virus Zaire. Virology **332:**406–417.
3. **Buchholz, U. J., S. Biacchesi, Q. N. Pham, K. C. Tran, L. Yang, C. L. Luongo, M. H. Skiadopoulos, B. R. Murphy, and P. L. Collins.** 2005. Deletion of M2 gene open reading frames 1 and 2 of human metapneumovirus: effects on RNA synthesis, attenuation, and immunogenicity. J. Virol. **79:**6588–6597.
4. **Buchholz, U. J., S. Finke, and K. K. Conzelmann.** 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. **73:**251–259.
5. **Collins, P. L., E. Camargo, and M. G. Hill.** 1999. Support plasmids and support proteins required for recovery of recombinant respiratory syncytial virus. Virology **259:**251–255.
6. **Collins, P. L., M. G. Hill, J. Cristina, and H. Grosfeld.** 1996. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. Proc. Natl. Acad. Sci. USA **93:**81–85.
7. **Conzelmann, K. K.** 2004. Reverse genetics of mononegavirales. Curr. Top. Microbiol. Immunol. **283:**1–41.
8. **Fearns, R., and P. L. Collins.** 1999. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. J. Virol. **73:**5852–5864.
9. **Feldmann, H., E. Mühlberger, A. Randolf, C. Will, M. P. Kiley, A. Sanchez, and H. D. Klenk.** 1992. Marburg virus, a filovirus: messenger RNAs, gene order, and regulatory elements of the replication cycle. Virus Res. **24:**1–19.
10. **Fowler, T., S. Bamberg, P. Möller, H. D. Klenk, T. F. Meyer, S. Becker, and T. Rudel.** 2005. Inhibition of Marburg virus protein expression and viral release by RNA interference. J. Gen. Virol. **86:**1181–1188.
11. **Groseth, A., H. Feldmann, S. Theriault, G. Mehmetoglu, and R. Flick.** 2005. RNA polymerase I-driven minigenome system for Ebola viruses. J. Virol. **79:**4425–4433.
12. **Hardy, R. W., and G. W. Wertz.** 2000. The Cys_3-His_1 motif of the respiratory syncytial virus M2-1 protein is essential for protein function. J. Virol. **74:**5880–5885.
13. **Hardy, R. W., and G. W. Wertz.** 1998. The product of the respiratory syncytial virus M2 gene ORF1 enhances readthrough of intergenic junctions during viral transcription. J. Virol. **72:**520–526.
14. **Martini, G. A., and R. Siegert (ed.).** 1971. Marburg virus disease. Springer, Berlin, Germany.
15. **Modrof, J., S. Becker, and E. Mühlberger.** 2003. Ebola virus transcription activator VP30 is a zinc-binding protein. J. Virol. **77:**3334–3338.
16. **Modrof, J., C. Moritz, L. Kolesnikova, T. Konakova, B. Hartlieb, A. Randolf, E. Mühlberger, and S. Becker.** 2001. Phosphorylation of Marburg virus VP30 at serines 40 and 42 is critical for its interaction with NP inclusions. Virology **287:**171–182.
17. **Mühlberger, E., B. Lötfering, H.-D. Klenk, and S. Becker.** 1998. Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. J. Virol. **72:**8756–8764.
18. **Mühlberger, E., M. Weik, V. E. Volchkov, H.-D. Klenk, and S. Becker.** 1999. Comparison of the transcription and replication strategies of marburg virus and Ebola virus by using artificial replication systems. J. Virol. **73:**2333–2342.
19. **Neumann, G., H. Feldmann, S. Watanabe, I. Lukashevich, and Y. Kawaoka.** 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J. Virol. **76:**406–410.
20. **Neumann, G., M. A. Whitt, and Y. Kawaoka.** 2002. A decade after the generation of a negative-sense RNA virus from cloned cDNA - what have we learned? J. Gen. Virol. **83:**2635–2662.
21. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
22. **Schnell, M. J., T. Mebatsion, and K. K. Conzelmann.** 1994. Infectious rabies viruses from cloned cDNA. EMBO J. **13:**4195–4203.
23. **Slenczka, W. G.** 1999. The Marburg virus outbreak of 1967 and subsequent episodes. Curr. Top. Microbiol. Immunol. **235:**49–75.
24. **Theriault, S., A. Groseth, G. Neumann, Y. Kawaoka, and H. Feldmann.** 2004. Rescue of Ebola virus from cDNA using heterologous support proteins. Virus Res. **106:**43–50.
25. **Towner, J. S., J. Paragas, J. E. Dover, M. Gupta, C. S. Goldsmith, J. W. Huggins, and S. T. Nichol.** 2005. Generation of eGFP expressing recombinant Zaire ebolavirus for analysis of early pathogenesis events and high-throughput antiviral drug screening. Virology **332:**20–27.
26. **Volchkov, V. E., V. A. Volchkova, E. Mühlberger, L. V. Kolesnikova, M. Weik, O. Dolnik, and H. D. Klenk.** 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science **291:**1965–1969.
27. **Weik, M., S. Enterlein, K. Schlenz, and E. Mühlberger.** 2005. The Ebola virus genomic replication promoter is bipartite and follows the rule of six. J. Virol. **79:**10660–10671.
28. **Weik, M., J. Modrof, H. D. Klenk, S. Becker, and E. Mühlberger.** 2002. Ebola virus VP30-mediated transcription is regulated by RNA secondary structure formation. J. Virol. **76:**8532–8539.
29. **Wu, H. N., Y. J. Lin, F. P. Lin, S. Makino, M. F. Chang, and M. M. Lai.** 1989. Human hepatitis delta virus RNA subfragments contain an autocleavage activity. Proc. Natl. Acad. Sci. USA **86:**1831–1835.